AD_____

Award Number: DAMD17-99-1-9095

TITLE: DNA Methylation Alterations in Breast Cancer

PRINCIPAL INVESTIGATOR: Fumiichiro Yamamoto, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
DNA Methylation Alterations in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9095

6. AUTHOR(S)
Fumiichiro Yamamoto, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-MAIL:
fyamamoto@burnham.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We proposed to investigate breast cancer-associated DNA methylation alterations using a technique named Methylation Sensitive Restriction Fragment Length Polymorphism (MS-AFLP) which we developed.

We performed NotI-MseI MS-AFLP using clinical specimens of normal and tumor breast DNA. We used both combinations of four NotI and four MseI primers with an additional selective residue at the 3' end (4x4 format) and combinations of four NotI with one additional residue and sixteen MseI with two additional selective residues (4x16 format). We have identified many bands that exhibited alterations in band intensity between normal and tumor breast DNA fingerprints. We have cut out the dried gel slices from those bands, eluted and PCR-amplified DNA fragments, and cloned into a plasmid vector. After transformation of *E. coli* bacteria, the nucleotide sequences of inserts were determined from several transformant clones. For some of the interesting bands, we have also transferred DNA from the DNA fingerprints by electroblotting onto nylon membranes, hybridized those membranes with fragment probes prepared from individual clones, and determined the identity of the fragments showing alterations.

From these studies, we have obtained results indicating the possible associations of homeotic genes and related genes in the pathogenesis of breast cancer.

14. SUBJECT TERMS
Breast Cancer, DNA methylation, genome scanning, genomics,
MS-AFLP, DNA fingerprinting

15. NUMBER OF PAGES
37

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Fumichiro Yamamoto 7/21/00

PI - Signature Date

Table of Contents

INTRODUCTION

The methylation of deoxycytosine at CpG dinucleotides is the most common form of post-replicative modification of DNA. The mechanisms regulating these modulations (the loss of the methylated status, i.e., hypomethylation, and the methylation of a previously unmethylated nucleotide, i.e., hypermethylation) are poorly understood. However, a hypothesis currently accepted is that these subtle alterations in DNA structure may contribute to the changes in chromatin structure, which occasionally result in the fine tuning of gene expression (1). DNA methylation has been shown to play a major role in such fundamental processes in cell biology as embryogenesis, cell differentiation and aging (2-7). In addition to the normal physiological roles of somatic DNA methylation, aberrations in the mechanisms that ensure its fidelity of perpetuation also play an important role in pathological processes, one of which is exemplified by cancer development and progression.

Cancer often results from a series of random accidents subjected to natural selection. Therefore, no two cases may be identical. Nonetheless, all cancers involve a disruption of normal restraints on cell proliferation and survival. Such a disruption may result from genetic and epigenetic alterations. Two routes exist which contribute to uncontrolled cell proliferation: activation of proto-oncogenes, and inactivation of tumor suppressor genes (8). For instance, changes associated with colorectal cancer include loss of APC (adenomatous polyposis coli), activation of Ki-*ras*, and loss of p53 (9,10). Activation of proto-oncogenes and inactivation of tumor suppressor genes result from either mutation(s) in coding sequence, aneuploidy (loss/gain of chromosomes and subchromosomal regions), gene amplification, or transcriptional changes. In addition to structural mutations affecting the genetic code, changes in the levels of cancer gene products can also result from alterations in the epigenetic code. Hypermethylation of CpG islands in the promoter region has been shown to be another important mechanism of inactivation through transcriptional controls, and has been closely correlated with the silencing of transcription of certain genes during tumorigenesis (11,12). For example, frequent loss of E-cadherin expression in some breast and prostate carcinomas was shown to result from hypermethylation of the promoter region (13). Hypermethylation was also reported for the promoter region of hMLH1, a mutator gene involved in hereditary non-polyposis colorectal cancer (HNPCC) and some sporadic gastrointestinal cancer (14).

Precise mapping of DNA methylation patterns in CpG islands is essential for understanding diverse biological processes. Several techniques have been developed to determine alterations in the epigenetic code. Some methods allow us to determine the degree of methylation of specific genes once their sequences are identified (15-18). In addition to the established method of digestion with methylation sensitive restriction enzymes followed by Southern hybridization or PCR, methylation-specific PCR (MSP) has recently been developed as a novel PCR assay for determining the methylation status of CpG islands (16). Using this technique, hypermethylation associated with transcriptional inactivation was reported in the

promoter regions of four important tumor suppressor genes in human cancer (p16 (19), p15 (20), E-cadherin (13), and von Hippel-Lindau (21). Techniques are also available for determining the degree of methylation in bulk DNA (22). However, there are few techniques that generate a comprehensive methylation profile of human genomic DNA (23-26).

We have developed a technique, called methylation sensitive-amplified fragment length polymorphism (MS-AFLP). MS-AFLP is an efficient and sensitive method that permits the evaluation of the spectrum of genetic and epigenetic alterations on a genomic scale. The technique utilizes the DNA fingerprinting advantages of the AFLP (27) and methylation sensitivity of restriction enzymes. We modified the AFLP to use a methylation sensitive restriction enzyme in place of methylation-insensitive *EcoRI*. We selected *NotI*, and performed *NotI-MseI* MS-AFLP experiments. Unlike *EcoRI*'s hexanucleotide recognition sites, *NotI* sites are octanucleotides (GCGGCCGC). The human genome is forty-nine percent G+C, and the CG dinucleotide is five-fold rarer than the expected G+C content (28). Therefore, sequences recognized by the *NotI* enzyme are rare. The total number of *NotI* sites is estimated to be between 30,000 and 100,000, and about 5,300 distinct sites are unmethylated and cleavable in certain tissues (29). This value is much smaller than that of *EcoRI* sites ($6x10^5$). In the *NotI* system only one additional nucleotide per primer is sufficient to obtain meaningful banding patterns, differing from the *EcoRI* system where at least three additional nucleotides are necessary for each primer in order to achieve meaningful banding patterns of the AFLP fingerprints. Accordingly, most of the *NotI* fragments are revealed by just 16 reactions (theoretically 663 bands per reaction (5,300x2/16)). When two (rather than one) additional nucleotides are added to one of the primers, the number of bands per reaction decreases to 166. Another advantage of using *NotI* is its methylation sensitivity. *NotI* does not cleave the site if the cytosine residue of CpG is methylated. Therefore, methylated and unmethylated *NotI* sites can be easily distinguished.

BODY

Proposed Task 1. To investigate breast cancer-associated DNA methylation changes (months 1-18).

1. NotI-MseI MS-AFLP will be performed with 10 sets of normal and tumor DNA from breast cancer patients

4x4 format using combinations of four NotI and four MseI primers with an additional selective residue (G, A, T, or C) at the 3' end (months 1-6)

4x16 format using four NotI with one additional residue and sixteen MseI with two additional selective residues (GG, GA, GT, GC, AG, AA, AT, AC, TG, TA, TT, TC, CG, CA, CT, or CC) (months 7-24)

We performed NotI-MseI MS-AFLP using 10 matched clinical specimens of normal and tumor breast DNA. We performed NotI-MseI MS-AFLP both in 4x4 format and in 4x16 format.

Proposed Task 2. To determine the identity of bands exhibiting consistent changes in breast cancers (months 2-36).

1. Alterations in band intensity will be identified, and DNA fragments will be cloned from individual bands showing alterations (months 2-20)
2. Plasmid clones containing the inserts exhibiting the alterations will be identified (months 3-24)
3. Nucleotide sequences will be determined (months 4-28)
4. BLAST search will be performed to identify the identical/homologous sequences in the database (months 5-32)
5. Important fragments will be mapped on chromosome using GeneBridge 4 Radiation Hybrid panel (months 6-36)
6. 5'- and 3'-RACE will be performed to clone the entire cDNAs for the sequences showing high homology with those deposited in EST database but not identical to the known genes (months 13-36)
7. RT-PCR will be performed to examine the effects of DNA methylation alterations on gene expression (months 19-36)

We have identified many bands that exhibited band intensity alterations between normal and tumor breast DNA fingerprints. We have cut out the dried gel slices from those bands, eluted and PCR-amplified DNA fragments, and cloned the fragments into pT-Adv plasmid vector by the T-A cloning method. After transformation of *E. coli* bacteria, plasmid DNA was prepared from several transformant clones, and the nucleotide sequences of the inserts were determined. Results are summarized in Table 1. For some of the interesting bands, we also transferred DNA from the DNA fingerprints onto nylon membranes by electroblotting, hybridized those membranes with the probes prepared from individual fragments from the sequence-verified clones, and determined the identity of the fragments showing alterations.

The most notable feature of Table 1, in addition to the presence of some genes with impact in cell growth (oncogenes and suppressor genes), is the high number of entries related to the homeobox genes and genes involved in the regulation of homeotic gene expression. Because

regulated cell growth and differentiation are the basis of development and cancer results from uncontrolled growth of undifferentiated cells, it is not difficult to speculate that activation/inactivation of certain homeotic genes may contribute to carcinogenesis. Actually, such examples have been reported (30-33). Therefore, our results demonstrated the capability of the *NotI-MseI* MS-AFLP method to identify and isolate the genes whose changes may be involved in cancer development and/or progression.

Table 1. Preliminary characterization of MS-AFLP bands in prostate, colon and breast cancers

No.	Frequency[1]	GenBank[2] number LOCUS[3] (band size, number of plasmid clones[4],
B1	- (8/10)	❐ cDNA c-0sg02 (256bp, 1/5), Cosmid U129A10 (248bp, 2/5)
B3	+ (4/10)	❐ zk16c05.r1 cDNA (493 bp, 1/7)
B4	- (7/10)	◆ ccr2, ccr5 and ccr6 genes and lactoferrin gene (616bp, 1/7)
B6	+ (8/8)	❐ Lung NbHL19W (339bp, 8/11), Z73359 (264bp, 1/11, 2.5e-11)
B8	- (9/10)	X12876 (280bp, 1/6, 3.2e-70)
B9	+ (5/9)	❐ Placenta 2 (3/8); cosmid B5E3 (1/8), ❐ yg96g09.r1 cDNA (1/8)
B10	+ (5/9)	❐ yf54a12.r1 cDNA (>515bp, 1/3)
B11	- (3/10)	◆ Ornithine decarboxylase (316bp, 1/3), ◆ antisecretory factor-1 (321bp, 1/3)
B12	+ (8/9)	◆ U05681 BCL3 (257bp, 3/4, 0.15)
B13	+ (3/9)	◆ X93086 Biliverdin-9 alpha reductase (58bp, 3/3, 5.4e-15)
B14	- (8/10)	❐ Heart NbHH19W (283bp, 1/3)
B15	- (5/10)	■ G2822174 **HOX A1- HOX A2** (169bp, 2/5, 8.6e-43)
B16	- (5/9)	❐ heart NbHH19W (284bp, 1/5), Cosmid 366D3 (256bp, 2/5)
B18	- (6/10)	❐ EST30769 Colon 1 (158bp, 1/1)
B20	- (6/10)	● D26155 **hSNF2α** (210bp, 3/4, 5.5e-44)
B21	+ (5/10)	❐ Testis NHT (293bp, 1/2)
B23	- (3/8)	❐ Brain 7B35H12 (123bp, 3/3)
B24	- (6/10)	◆ TAXREB67 (168bp, 1/3, 1.3e-45), cosmid U27H1 (201bp, 1/3)
B25	- (6/10)	■ U57052 **HOX B13** (212bp, 5/5, 1.4e-77)
B26	- (8/10)	◆ D13644 KIAA0019 gene (288bp, 1/5, 3.6e-102)
B27	- (5/10)	■ X99894 **IPF-1** (450bp, 3/5, 2.1e-172)
B28	- (6/9)	◆ D28596 **c-maf** (100bp, 1/2, 1.9e-13)
B30	+ (1/3)	❐ yj84a11.s1 cDNA (390bp, 1/3), Alu-Sb subfamily (434bp, 1/3)

1. +; stronger band intensity in tumor tissue rather than in normal tissue (hypomethylation), -; stronger band intensity in normal tissue rather than in tumor tissue (hypermethylation), (number of tumors where the difference was observed/number of tumors analyzed).
2. In addition, there were 36 DNA fragments from 35 *NotI-MseI* MS-AFLP fingerprint bands exhibiting tumor-specific intensity changes without homology with GenBank sequences (14 from prostate, 3 from colon and 19 from breast cancers).
3. Probabilities given in the GenBank search.
4. Number of plasmid clones with the same sequence/number of clones sequenced.
5. Three sequences from prostate and 8 from breast tissues represented anonymous CpG island DNA clones, respectively.
6. Symbol ◆ indicates genes previously characterized, ■ homeotic genes, ● genes involved in regulation of homeotic gene expression, and ❐ anonymous expressed sequences. The genes with known roles in cell proliferation are highlighted in bold.
Sequences that have been confirmed by the Southern hybridization to exhibit the observed changes are shown underlined. In addition, our sequence analysis of 64 randomly chosen *NotI-MseI* MS-AFLP cloned fragments showed that approximately one-fourth of the fragments were either identical or closely related to the sequences deposited in the EST database. The table has been simplified by eliminating anonymous and repetitive sequences.

KEY RESEARCH ACCOMPLISHMENTS

1. We have scanned DNA methylation alterations at the NotI landmarks over the entire genome of normal and tumor breast cancer tissue DNA.
2. We have identified several DNA fragments derived from homeotic genes and related genes among the bands that exhibited band intensity, suggesting the possible association of homeotic genes with breast carcinogenesis.

REPORTABLE OUTCOMES

1. We have written and submitted a manuscript that describes the technical details of NotI-MseI MS-AFLP technique.
2. We have also summarized our findings of widespread DNA methylation alterations of homeotic genes and related genes in cancer and submitted it as a short communication.

CONCLUSIONS

We have already finished Task 1 of the proposal. We have determined the identity of several sequences that exhibited DNA methylation alterations. We have determined the nucleotide sequences of fragments cloned into plasmid from dozens of other bands, however, the determination of real sequences that exhibit the alteration remained to be done. We have decided not to pursue chromosomal mapping since 97% of the human genome has already been sequenced and we do not see the point of experimentally determining the loci of DNA fragments using GeneBridge Radiation Hybrid panel. We have not yet initiated the last two works in Task 2.

REFERENCES

1. Jones, P.L. *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**, 187-191 (1998).

2. Counts, J.L. & Goodman, J.I. Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell* **83**, 13-15 (1995).

3. Jones, P.A. & Laird, P.W. Cancer epigenetics comes of age. *Nat Genet* **21**, 163-167 (1999).

4. Kass, S.U., Pruss, D. & Wolffe, A.P. How does DNA methylation repress transcription? *Trends Genet* **13**, 444-449 (1997).

5. Baylin, S.B., Herman, J.G., Graff, J.R., Vertino, P.M. & Issa, J.P. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* **72**, 141-196 (1998).

6. Cedar, H. & Verdine, G.L. Gene expression. The amazing demethylase [news; comment]. *Nature* **397**, 568-569 (1999).

7. Issa, J.P. *et al.* Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* **7**, 536-540 (1994).

8. Weinberg, R.A. The molecular basis of oncogenes and tumor suppressor genes. *Ann N Y Acad Sci* **758**, 331-338 (1995).

9. Vogelstein, B. & Kinzler, K.W. The multistep nature of cancer. *Trends Genet* **9**, 138-141 (1993).

10. Kinzler, K.W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170 (1996).

11. Laird, P.W. & Jaenisch, R. DNA methylation and cancer. *Hum Mol Genet* **3**, 1487-1495 (1994).

12. Klein, C.B. & Costa, M. DNA methylation, heterochromatin and epigenetic carcinogens. *Mutat Res* **386**, 163-180 (1997).

13. Graff, J.R. *et al.* E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* **55**, 5195-5199 (1995).

14. Kane, M.F. *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* **57**, 808-811 (1997).

15. Clark, S.J., Harrison, J., Paul, C.L. & Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* **22**, 2990-2997 (1994).

16. Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. & Baylin, S.B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* **93**, 9821-9826 (1996).

17. Gonzalgo, M.L. & Jones, P.A. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res* **25**, 2529-2531 (1997).

18. Xiong, Z. & Laird, P.W. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* **25**, 2532-2534 (1997).

19. Jarrard, D.F. *et al.* Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. *Genes Chromosomes Cancer* **19**, 90-96 (1997).

20. Herman, J.G., Jen, J., Merlo, A. & Baylin, S.B. Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res* **56**, 722-727 (1996).

21. Waber, P.G., Lee, N.K. & Nisen, P.D. Frequent allelic loss at chromosome arm 3p is distinct from genetic alterations of the Von-Hippel Lindau tumor suppressor gene in head and neck cancer. *Oncogene* **12**, 365-369 (1996).

22. Singhal, R.P., Mays-Hoopes, L.L. & Eichhorn, G.L. DNA methylation in aging of mice. *Mech Ageing Dev* **41**, 199-210 (1987).

23. Hatada, I., Sugama, T. & Mukai, T. A new imprinted gene cloned by a methylation-sensitive genome scanning method. *Nucleic Acids Res* **21**, 5577-5582 (1993).

24. Huang, T.H. *et al.* Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. *Cancer Res* **57**, 1030-1034 (1997).

25. Huang, T.H., Perry, M.R. & Laux, D.E. Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet* **8**, 459-470 (1999).

26. Xiong, L.Z., Xu, C.G., Saghai Maroof, M.A. & Zhang, Q. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. *Mol Gen Genet* **261**, 439-446 (1999).

27. Vos, P. *et al.* AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407-4414 (1995).

28. McClelland, M. & Ivarie, R. Asymmetrical distribution of CpG in an 'average' mammalian gene. *Nucleic Acids Res* **10**, 7865-7877 (1982).

29. Yoshikawa, H. *et al.* Chromosomal assignment of human genomic NotI restriction fragments in a two-dimensional electrophoresis profile. *Genomics* **31**, 28-35 (1996).

30. Maulbecker, C.C. & Gruss, P. The oncogenic potential of deregulated homeobox genes. *Cell Growth Differ* **4**, 431-441 (1993).

31. Ee, H.C., Erler, T., Bhathal, P.S., Young, G.P. & James, R.J. Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. *Am J Pathol* **147**, 586-592 (1995).

32. Vider, B.Z. *et al.* Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. *Biochem Biophys Res Commun* **232**, 742-748 (1997).

33. Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F. & Beck, F. Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* **386**, 84-87 (1997).

Title

NotI-MseI Methylation Sensitive-Amplified Fragment Length Polymorphism (MS-AFLP) for DNA methylation analysis of human cancers

Authors

Fumiichiro Yamamoto[1]*, Jose Luis Soto[1], Miyako Yamamoto[1], Takao Sekiya[2], and Manuel Perucho[1]

[1]The Burnham Institute, La Jolla Cancer Research Center, La Jolla CA 92037, USA

[2]Oncogene Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan

* Author to whom correspondence should be addressed:

Fumiichiro Yamamoto, Ph.D.

The Burnham Institute, La Jolla Cancer Research Center

10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA.

Phone: 858-646-3116, FAX: 858-646-3173

E-mail: fyamamoto@burnham.org

Running Title

Methylation sensitive AFLP

Abstract

We have applied a methylation sensitive restriction endonuclease NotI to the existing Amplified Fragment Length Polymorphism (AFLP) method and developed NotI-MseI Methylation Sensitive-AFLP (MS-AFLP). NotI-MseI MS-AFLP allows the genome-wide analysis of DNA methylation alterations at the NotI sites scattered over the genome. Hypermethylation and hypomethylation are visualized by the decrease and increase in the band intensity of the DNA fingerprints. Identification of consistent changes can be facilitated through parallel electrophoresis of multiple samples. DNA fragments exhibiting alterations can be cloned from fingerprint bands by amplification of gel-eluted DNA with the same pair of primers used for fingerprint presentation. NotI-MseI MS-AFLP offers a reliable method of studying the alterations in DNA methylation in the human genome.

Introduction

The cytosine methylation of CpG dinucleotide sequence is the most common form of post-replicative modification of genomic DNA in higher eukaryotes. Using palindromic nature of the sequence, methylation pattern can be transmitted to the daughter strands by the *maintenance* methylation. Although the underlying mechanisms are poorly understood, somatic *de novo* methylation and demethylation can also occur. Regions containing a high density of CpG dinucleotides are called CpG islands, and they are predominantly associated with coding DNA. In the past years, DNA methylation of CpG islands has been proven to be one important mechanism controlling gene expression. CpG islands of constitutively expressed genes are unmethylated in the germ line and in all somatic cells (1). On the other hand, DNA methylation of CpG islands plays a regulatory role in the tissue-specific gene expression (2). Silencing of one X chromosome (imprinting) is also tightly associated with alterations in the methylation status of CpG islands (3,4). DNA hypermethylation seems to be very important in the aging process also (5). A hypothesis currently accepted is that the subtle alterations in DNA structure may contribute to the changes in chromatin structure, resulting in repression of gene expression (6). In addition to the normal physiological roles of somatic DNA methylation, aberrations in the mechanisms that ensure its fidelity of perpetuation also play an important role in pathological processes, one of which is exemplified by cancer development and progression.

All cancers involve a disruption of normal restraints on cell proliferation and survival. Such a disruption may result from genetic and epigenetic alterations. Activation of proto-oncogenes and inactivation of tumor suppressor genes contribute to the uncontrolled cell proliferation, and these result from either mutation in coding sequence, aneuploidy (loss/gain of chromosomes and/or subchromosomal regions), gene amplification, or transcriptional changes. In all cases, the final consequence is having either more or less of the corresponding functional gene products or altered activity of these products (7). Hypermethylation of CpG islands in the promoter region is an important mechanism of inactivation through transcriptional controls (8,9). CpG island hypermethylation has been closely correlated with the silencing of transcription of certain genes

during tumorigenesis. For example, frequent loss of E-cadherin expression in some breast and prostate carcinomas was shown to result from hypermethylation of the promoter region (10). Methylation of NotI sites at D17S5 on chromosome 17p13.3 was found to be tumor-specific in colon, brain, and kidney tumors (11-13). Somatic hypermethylation of the CpG island was frequently observed in the receptor gene for endothelin-1 (EDNRB) in prostate cancer tissues (14). Because endothelin-1 receptor mediates clearance of this potent vasoconstrictor and inhibits secretion, decreased expression of the receptors by hypermethylation of the promoter may be advantageous for cancer progression *in vivo*. Hypermethylation was also reported for the promoter region of hMLH1, a mutator gene involved in hereditary non-polyposis colorectal cancer (HNPCC) and some sporadic gastrointestinal cancer (15).

Methylation analysis has mostly been conducted on the promoter regions of genes important for growth regulation. However, precise mapping of DNA methylation patterns in CpG islands on a genomic scale is essential for understanding diverse biological processes. We have developed a technique, called NotI-MseI Methylation Sensitive-Amplified Fragment Length Polymorphism (MS-AFLP). NotI-MseI MS-AFLP is an efficient and sensitive method that permits the genome-wide evaluation of the spectrum of epigenetic alterations (and probably genetic alterations also), as well as the identification and cloning of DNA fragments that exhibit alterations. The technique utilizes the DNA fingerprinting advantages of the AFLP (16) and methylation sensitivity of the rare-cutter NotI endonuclease, and can be applied to the analysis of complex genome of humans.

Materials and Methods

1. Materials

DNA from one matched pair of normal and metastasized tumor breast tissues, four matched pairs of normal and primary tumor prostate tissues, and five matched pairs of normal and primary tumor lung tissues was used in the study. The breast and prostate tissues were obtained from the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Normal and tumor lung tissue DNA was kindly provided by Dr. Yoshinori Murakami (Oncogene Division, NCCRI, Japan). Restriction endonuclease NotI and T_4 DNA ligase were purchased from Boehringer Mannheim (Indianapolis, IN). Restriction endonuclease MseI and T_4 polynucleotide kinase were obtained from NE Biolabs (Beverly, MA), and AmpliTaq DNA polymerase was purchased from Perkin-Elmer (Foster City, CA). Takara ExTaq DNA polymerase was from PanVera (Madison, WI). ^{32}P-gamma Easytide adenosine 5'-triphosphate (^{32}P-γATP) was purchased from NEN–Life Science Products (Boston,MA). Oligonucleotides were custom-synthesized at Genset (La Jolla, CA) and at Sigma-Genosys (The Woodlands, TX). NotI and MseI adaptors were equimolar mixtures of two oligonucleotides. The nucleotide sequences of the two oligonucleotides for the MseI adaptor were 5'-GACGATGAGTCCTGAG and 5'-TACTCAGGACTCAT, and these sequences were identical to those used by Vos et al. (16). The nucleotide sequences of the oligonucleotides for the NotI adaptor were 5'-GACTGCGTACCGGCCGC and 5'-GGCCGGTACGCAGTCTAC, and these sequences were modified from their EcoRI adaptor sequences. These pairs of oligos were mixed, heated at 65^{0}C for 15 min, and kept at 37^{0}C overnight for annealing before use. Primers used for PCR amplification were NotI+N (5'-GACTGCGTACCGGCCGC+N), MseI+N (5'-GATGAGTCCTGAGTAA+N), and MseI+CN (5'-GATGAGTCCTGAGTAA+CN) adaptor primers. NotI+G adaptor primer labeled at the 5' end with TAMRA fluorescent dye, 5'-TAMRA-GACTGCGTACCGGCCGCG, was custom synthesized at Genset.

2. NotI-MseI MS-AFLP Method

Experimental protocol was modified from the original AFLP protocol (16). Five hundred nanograms of genomic DNA were digested overnight at 37^{0}C in 25 µl of 1xNotI digestion buffer with 5 units of NotI and 2 units of MseI. Then 17 µl of 1mM Tris-HCl, 1mM EDTA buffer (1-1TE), 5 µl of 10xT_4 DNA ligase buffer, 1.25 µl each of 5 pmol/µl NotI and 50 pmol/µl MseI adaptors, and 1 unit of T_4 DNA ligase, were added, and the DNA was incubated overnight at 16^{0}C. The ligated DNA was incubated at 37^{0}C for 2-6 hrs, heat-denatured at 70^{0}C for 20 min to inactivate the enzymes, and diluted to adjust the concentration to 1 ng/µl.

For the radioactive NotI-MseI MS-AFLP experiments, one of the primers was radiolabeled at the 5' end using T_4 polynucleotide kinase and ^{32}P-γATP. NotI primer was radiolabeled in all the experiments except one where MseI primer was labeled. Six nanograms of NotI primer and 30 ng of MseI primer were used for PCR with adaptor ligated genomic DNA template in 20 µl of 1xAmpliTaq DNA polymerase buffer, 0.4mM dNTP, with 1 unit of AmpliTaq DNA polymerase. Various amounts of adaptor ligated DNA (2 ng, 400 pg, 80 pg, 16 pg) was used in the experiments with lung DNA. 5 ng of adaptor ligated DNA was used in the breast and prostate experiments. Two hundred µl tubes were used and placed in a 96 well-type DNA Thermal Cycler. PCR protocol was the following: 72 ^{0}C for 30 sec, 94 ^{0}C for 30 sec, 36 cycles of 94 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, and 72 ^{0}C for 2 min, followed by incubation at 72 ^{0}C for 10 min. The samples were kept at 10 ^{0}C until the reaction was terminated by the addition of 25 µl of loading dye solution. After 3 min of heat denaturing followed by a quick chill on ice, an aliquot (4 µl) from each reaction was loaded onto a SEQUAGEL-6 denaturing gel. After electrophoresis, the gel was dried and then exposed to an X-ray film.

For the fluorescent MS-AFLP experiments, thirty nanograms each of fluorescent NotI primer and unlabeled MseI primer and ten nanograms of template DNA were used in 20 µl of 1 X ExTaq DNA polymerase buffer, 0.4 mM dNTP, with 1 unit of ExTaq DNA polymerase. The same PCR protocol described above was used. PCR products were added with 2 µl of 3M sodium acetate (pH 5.2) and 50 µl of 95% ethanol, incubated at 4 ^{0}C for 2 hours, and then precipitated. After washed

with 70% ethanol and air dried, the pellets were resuspended in 6 μl of dye containing 5:1 ratio of formamide: Blue Dextran-EDTA. The samples were then loaded onto an ABI Prism 377 automatic DNA Sequencer and electrophoresed according to the manufacturer's protocols.

Results

Theoretical basis of NotI-MseI MS-AFLP

We modified the AFLP to use methylation sensitive NotI restriction enzyme in place of EcoRI. Unlike hexanucleotide EcoRI recognition sites, NotI sites are octanucleotides (GCGGCCGC). The human genome is forty-nine percent G+C, but the CpG dinucleotide is five-fold rarer than the expected G+C content (17). Therefore, sequences recognized by the NotI enzyme are rare. The total number of NotI sites is calculated to be around $8x10^4$. In certain tissues, however, only approximately 5,300 NotI sites are unmethylated and cleavable (18). This value is much smaller than the estimated $8x10^5$ cleavable EcoRI sites. Therefore, only one additional nucleotide per primer is sufficient in the NotI system to obtain meaningful banding patterns of AFLP fingerprints of the human genome, differing from the EcoRI system where at least three additional nucleotides are necessary for each primer. Accordingly, most of the NotI fragments (NotI-MseI and NotI-NotI) can be revealed by just 16 reactions (theoretically 5,300x2/16=663 bands per reaction). When two, rather than one, additional nucleotides are added at one of the primers, the number of bands per reaction decreases to 166. Therefore, the alterations in the methylation status of NotI sites can be easily distinguished by the changes in band intensity in DNA fingerprints. The NotI-MseI MS-AFLP method is schematically shown in Figure 1.

Reproducibility of the NotI-MseI MS-AFLP fingerprinting

We examined the reproducibility of NotI-MseI MS-AFLP. After digestion with NotI and MseI, human genomic DNA was ligated with adaptors and used as a template for MS-AFLP DNA fingerprint presentation. In order to determine the amount of template necessary to obtain reproducible results, titration experiments were performed. ^{32}P-radiolabeled NotI+G primer was used in combination with unlabeled MseI+C primer. Reactions were duplicated in 2 different tubes, and were electrophoresed in parallel. Adaptor ligated normal (N) lung DNA was serially diluted, and the same 5 μl containing different amounts of DNA was used for the MS-AFLP in 20 μl reactions. Reaction products were analyzed through denaturing gel electrophoresis. Results are

shown in the left panel of Figure 2. Two separate reactions using the same adaptor-ligated DNA produced essentially identical banding patterns when 2 ng of template DNA was used. When the amount of template was reduced to 400 pg, sporadic changes in band intensity began to appear. When the amount was further reduced to 80 pg and 16 pg, banding patterns varied significantly between the duplicate reactions. We concluded that at least 2 ng of human genomic DNA (approximately 200 cell equivalents = 400 genome equivalents) was needed to obtain reproducible results.

DNA fingerprints, produced with five pairs of normal (N) and tumor (T) genomic DNA from lung cancer patients, were then examined. The same NotI+G and MseI+C primers were used. All the reactions were duplicated. Results are shown in the right column of Figure 2. Two separate reactions using the same DNA template again produced essentially identical banding patterns. Cases 1 through 4 showed several somatic alterations, whereas few alterations were observed in case 5. Arrows indicate possible tumor-specific alterations.

Combinatorial MS-AFLP using adaptor primers with additional nucleotides

In the above experiments, only one pair of primer combination was used. In the next experiment, we used all sixteen combinations (4x4) of adaptor primers with one additional nucleotide each at their 3' ends. Five nanograms of adaptor ligated DNA from normal breast (N) and metastatic breast carcinoma (M) from the same individual was used as templates. ^{32}P-radioabeled NotI+N primers were used in combination with unlabeled MseI+N primers. The N denotes any one of G, A, T, and C nucleotides. Results are shown in Figure 3. The combinations of primers used are indicated on the top of the gel. Although some combinations gave better banding patterns than others, basically all the primer combinations worked. One reaction, NotI+T and MseI+A reaction with metastasized DNA, failed to give a good quality fingerprint from unknown cause(s).

We estimated the total numbers of bands exhibited by the 16 NotI-MseI MS-AFLP fingerprints to be 3,200 (200x16). This value was lower than the expected total number 12,600 (5,300x2).

Possible reasons for this discrepancy include: 1) some bands were represented by more than one DNA fragment, 2) some fragments with larger sizes or complicated sequence were not amplified, and 3) some amplified fragments were too small and ran out of the gel. Nevertheless, the number of different bands obtained seemed to be sufficient for a near saturation scanning of the genome. This number may be doubled to 6,400 by the use of two additional nucleotides at the 3' end of one of the primers as will be discussed below.

Multiple somatic fingerprint alterations were observed in this experiment alone, and several examples are indicated with up and down arrowheads in the figure. This demonstrates the potential of the NotI-MseI MS-AFLP technique that generated a large number of potentially interesting somatic epigenetic alterations in breast cancer from this single experiment. An additional property of the technique is that the dried gels can be stored at room temperature for months and even years, and used later to isolate the relevant bands. At the same time, the experiment illustrates another important property of the technique, its ability to obtain a panoramic view of the alterations undergone by the tumor cells.

We next compared the results obtained from NotI-MseI MS-AFLP using MseI primer with an additional nucleotide and those obtained using MseI primers with two additional nucleotides. DNA from four sets of matched normal and tumor prostate tissues was used in the experiment. ^{32}P-radiolabeled NotI+G primer was used in combination with unlabeled MseI+C for all four sets of DNA, and with unlabeled MseI+CG, MseI+CA, MseI+CT, or MseI+CC for the fourth set of DNA. Results are shown in Figure 4. Except for a few bands that were present in one of the four reactions but absent in the MseI+C reaction, the MseI+C fingerprint basically exhibited the sum of the bands produced by those four reactions. The result also showed that many bands in the MseI+C fingerprint were in fact composed of several comigrating bands. Therefore, the use of two additional nucleotides in the primers can be exploited to increase the total number of DNA fragments that can be analyzed and to increase the specificity of identification of sequences with alterations. The fact that the consistent alterations observed in 4 cases of prostate tumors were also

identified in one of the four primer combinations clearly demonstrated that those observed differences in band intensity were not an artifact but rather genuine sequence-dependent alterations.

Preferential amplification of NotI-MseI fragments over MseI-MseI fragments in NotI-MseI MS-AFLP

In addition to the advantage that a small amount of DNA template is sufficient, the PCR-based MS-AFLP may also offer a relatively easy cloning method of DNA fragments from fingerprint bands. This is true, however, only when NotI-NotI and NotI-MseI fragments are preferentially amplified. In the above experiments, we used ^{32}P radiolabeled NotI adaptor primers. MseI endonuclease cleaves at the TTAA sequence, and this tetranucleotide sequence is much more frequently found in the genome than the octanucleotide NotI sequence. Therefore, it was possible that in addition to the radiolabeled NotI-MseI and NotI-NotI fragments that sensitized the X-ray film, there might have been thousand times more of the unlabeled MseI-MseI fragments in the background. In order to examine this possibility, we performed MS-AFLP experiments using the same primer combination but using ^{32}P radiolabeled MseI adaptor primers and compared with the results from the MS-AFLP experiments with ^{32}P radiolabeled NotI adaptor primers. Results are shown in Figure 5. The two leftmost and the two rightmost lanes show the two pairs of MS-AFLP fingerprints using the same combinations of primers but labeled at different primers. The two lanes exhibited quite similar, though not identical, results that implicated that NotI-MseI (and NotI-NotI?) fragments were selectively amplified over MseI-MseI fragments under the PCR conditions employed.

Fluorescent NotI-MseI MS-AFLP

One advantage of using radioactive tags for NotI-MseI MS-AFLP is that it allows direct cloning of DNA fragments after the identification of alterations. However, use of fluorescent markers offers a different kind of advantage. They are safer, and this safe feature is important in clinical settings. Simultaneous usage of multiple dyes with different absorption and emission

ranges is possible. Therefore, we investigated the conditions for labeling the MS-AFLP products with a fluorescent primer, and examined the sensitivity of the detection. Highly reproducible fingerprint profiles were obtained using the protocol detailed in the Material and Methods section. Results from the fluorescent MS-AFLP experiment using a pair of normal and tumor DNA from a lung cancer case5, which exhibited few alterations using a radiolabeled primer, are shown in Figure 6A. Almost identical peak profiles were obtained. A few examples of the alterations observed in cases 2 and 4 are shown in Figures 6B (increased peak intensity in tumor) and C (decreased peak intensity in tumor).

Discussion

Precise mapping of DNA methylation patterns in CpG islands is essential for understanding diverse physiological and pathological biological processes. Several techniques have been developed to determine DNA methylation alterations of specific genes once their sequences are identified (19-22). In addition to the established methods of digestion with methylation sensitive restriction enzyme(s) followed by Southern hybridization or PCR, methylation-specific PCR (MSP) has been developed as a novel PCR assay for determining the methylation status of CpG islands (20). Using this technique, hypermethylation associated with transcriptional inactivation was reported in the promoter regions of four important tumor suppressor genes in human cancer (p16 (23), p15 (24), E-cadherin (10), and von Hippel-Lindau (25)). High sensitivity methylation mapping is possible by the sodium bisulfite modification-DNA sequencing method (19). Techniques are also available for determining the degree of methylation in bulk DNA (26). However, there are few techniques that generate a comprehensive methylation profile of human genomic DNA. Three scanning techniques are currently available for DNA methylation analysis: methylation-sensitive restriction fingerprinting (MSRF) (27), restriction landmark genomic scanning for methylation (RLGS-M) (28), and amplification of restriction polymorphism (29).

The MSRF method employs the principle of the AP-PCR approach. After digesting genomic DNA with MseI alone or MseI and methylation sensitive BstUI, the DNA is PCR-amplified using a specially designed pair of primers with short arbitrary 10-mers sequences attached to the flanking BstUI recognition sequence (CGCG). The PCR products are electrophoresed and banding patterns compared between the MseI digestion alone and the MseI/BstUI double digestion. Although this approach has successfully identified two breast carcinoma-specific hypermethylation fragments (HBC-1 and 2), scanning an entire genome in an organized manner remains an impossible task when using this method with arbitrary primer sequences. RLGS-M, another scanning technique, allows genomic scanning for changes in methylation at NotI sites. Because genomic DNA is directly end-labeled in RLGS-M, different from PCR-based techniques, the ratio of all the fragments remains unchanged. Moreover, the entire fingerprints can be obtained from one reaction

displayed on a two-dimensional gel. However, two-dimensional gel electrophoresis-based RLGS is complicated, and is not suitable for simultaneous analyses of multiple specimens although some success has been reported using a specially designed apparatus (30,31). Other shortcomings are that RLGS-M requires microgram quantities of good quality DNA to visualize individual genomic DNA fragment spots, and cloning of the DNA fragments are extremely difficult. Similar to NotI-MseI MS-AFLP, amplification of restriction polymorphism method utilizes the AFLP technique and a methylation sensitive restriction enzyme. Different combinations of enzymes (EcoRI and HpaII/MspI) are used, however.

The NotI-MseI MS-AFLP method is superior to other scanning techniques for DNA methylation alterations in several aspects. MSRF is similar to MS-AFLP in that the alterations are detected in one-dimensional denaturing sequencing gels, and the amplified products are relatively small DNA fragments representing anonymous genomic sequences. The advantage of our method is its superior reproducibility. The larger number of bands are amplified and the higher ratio of band/background noise is obtained (see Figures 2 through 5). Another advantage of the NotI-MseI MS-AFLP approach is that it may be more benefited from the Human Genome Project effort since the positions of the NotI sites in the genome will be easily identified compared with more abundant BstUI sites used in the MSRF approach. Therefore, with a defined NotI map, NotI-MseI MS-AFLP is advantageous for the systematic comparative screening of different situations. Compared with RLGS-M, MS-AFLP is clearly superior in several aspects. First, MS-AFLP requires much less template DNA. We usually use 5 ng of DNA for one MS-AFLP experiment with one pair of primers. If 16 reactions are performed by 4x4 format, a total of 80 ng of DNA is needed. Pre-amplification of the adaptor-ligated DNA fragments with universal primers without additional nucleotide at the 3' end before fingerprint presentation may reduce even more the total amount of template (data not shown). Moreover, simultaneous analysis of multiple samples is easily performed by parallel electrophoresis, which also facilitates the detection of consistent differences in band intensity. More importantly, in contrast to RLGS-M, MS-AFLP permits the single step cloning of the fingerprint bands from the gel by PCR with the adaptor primers. The amplification

of restriction polymorphism method is advantageous in its easy confirmation of DNA methylation alterations by side-by-side examination of the EcoRI/HpaII and EcoRI/MspI DNA fingerprints since both HpaII and MspI recognize the same CCGG sequence but differ in their sensitivity to the methylated sequence. The method is quite powerful, however, the use has been limited to the genomic analysis of organisms with relatively small genome sizes (32). This is also true for the regular AFLP experiments since the number of EcoRI sites is too large in the human genome, and the conditions to obtain reproducible results are tricky.

Using the NotI-MseI MS-AFLP method, we have analyzed DNA methylation alterations associated with tumorigenesis. Among those bands that exhibited alterations in cancer, we identified sequences from multiple homeobox genes and genes involved in the regulation of homeotic gene expression (F. Yamamoto et al. manuscript submitted for publication). Because homeoproteins are transcription factors that direct embryogenesis and cell differentiation, alterations of homeobox gene function may trigger a cascade of changes in gene expression. These changes may result in a cell with a less differentiated and less positionally restricted phenotype. These results, therefore, may demonstrate the ability of our approach to detect somatic epigenetic alterations relevant to neoplastic transformation. Although NotI-MseI MS-AFLP theoretically detects not only the changes in DNA methylation but also (sub)chromosomal gains/losses, most, if not all, of the alterations identified so far turned out to be the results of DNA methylation alterations.

In addition to the radioactive NotI-MseI MS-AFLP, we have also attempted the fluorescent NotI-MseI MS-AFLP. Since the conditions of PCR amplification, gel electrophoresis, and the method of detection were different, it seemed to be rather difficult, if not impossible, to correlate all the individual bands in radioactive MS-AFLP fingerprints and the individual peaks in fluorescent MS-AFLP electropherograms. Nonetheless, there was a clear positive correlation between the number of alterations in radioactive MS-AFLP and the number of alterations in fluorescent MS-AFLP, suggesting that fluorescent MS-AFLP is also useful in detecting the differences. Transformation of the gel electrophoresis-based NotI-MseI MS-AFLP into a hybridization-based

technique using DNA microarrays in the future may facilitate the determination of the identity of alterations. In conclusion, NotI-MseI MS-AFLP provides another method of DNA methylation analysis in both research and clinical settings.

Acknowledgments

We thank Fumiya Yamamoto for editing the manuscript and preparing the figures. We also thank Tomoko Maruyama for technical assistance. This work was supported by grants from the California Breast Cancer Research Program (4JB144) and from the Department of Defense Breast Cancer Research Program (DAMD17-99-1-9095) to FY, and a grant from NIH (5R01CA63585-07) to MP. FY, JLS, and MP were Foreign Research Fellows of the Foundation for Promotion of Cancer Research, Tokyo. Correspondence and requests for materials should be addressed to FY (e-mail: fyamamoto@burnham.org).

Figure legends

Figure 1. Schematic representation of the NotI-MseI MS-AFLP method

Unmethylated NotI sites are cleaved by NotI, NotI ends are ligated with NotI adaptors, and the adaptor-ligated NotI-MseI (and NotI-NotI) DNA fragments are amplified. Methylated sites, however, are resistant to cleavage, and no amplification is observed. By using primers with additional nucleotide(s) at the 3' end for PCR, only selected populations of DNA fragments are amplified, reducing the number of bands. Hypermethylation (and subchromosomal loss) and hypomethylation (and subchromosomal gains) are indicated by the decreased and increased band intensity of MS-AFLP fingerprints.

Figure 2. NotI-MseI MS-AFLP fingerprints of lung cancer

The results of NotI-MseI MS-AFLP experiments with one pair of primers (NotI+G and MseI+C) are shown. Left: Different amounts of adaptor ligated template DNA was used for the NotI-MseI MS-AFLP to determine the minimal amount of DNA required for reproducible results. Reactions were duplicated and the results are shown with 1 and 2. Right: Five pairs of the matched normal and tumor lung tissue DNA was analyzed by the NotI-MseI MS-AFLP. N and T denote normal and tumor tissue DNA, respectively. The up and down arrows indicate the bands that exhibited an increase and decrease in band intensity.

Figure 3. The NotI-MseI MS-AFLP fingerprints obtained using all the sixteen combinations of primers with one additional nucleotide each

DNA from normal breast (N) and metastatic breast carcinoma (M) from a patient was used for NotI-MseI MS-AFLP using 16 combinations of primer pairs with one additional nucleotide at the 3' end. The added nucleotide residues are shown above the lanes. Examples of prominent bands showing increases or decreases in band intensity in the tumor are indicated by up and down arrowheads, respectively.

Figure 4. Comparison between fingerprints obtained from the 4x4 format with those of the 4x16 format

Normal (N) and tumor (T) DNA from four prostate cancer patients (1P-4P) was used to compare the results from the 4x4 format and those from the 4x16 (two additional nucleotides were added to MseI primers). Three bands showing intensity changes in four prostate samples are indicated by open arrowheads. The adaptor-ligated DNA from case 3P was prepared separately from other samples. That is why the overall pattern of 3P fingerprint was somewhat different from the other three fingerprints. Surprisingly, however, the consistent alterations observed in the other fingerprints were also found in 3P fingerprint, suggesting that the alterations can be identified so far as both normal and tumor samples are processed in parallel.

Figure 5. Comparison of the NotI-MseI MS-AFLP fingerprints produced from reactions using ^{32}P radiolabeled NotI adaptor primers and ^{32}P radiolabeled MseI adaptor primers

Results from NotI-MseI MS-AFLP experiment using the same primer combinations but labeled at either NotI or MseI primers are shown in the two leftmost and two rightmost lanes. PCR products were analyzed by parallel electrophoresis.

Figure 6. Fluorescent NotI-MseI MS-AFLP fingerprints

Fluorescently labeled NotI+G primer and unlabeled MseI+C primer were used for NotI-MseI MS-AFLP, and the results were analyzed using an automatic DNA sequencer. A: Fluorescent NotI-MseI MS-AFLP electropherograms are shown of a pair of normal (N) and tumor (T) DNA from lung cancer case 5, which exhibited few alterations in the radioactive MS-AFLP experiments (see Figure 2). Almost identical peak profiles were obtained. B: Several examples of increased peaks observed in the electropherograms of cases 2 and 4 are shown. C: Several examples of decreased peaks are shown.

References

1. Bird, A., Taggart, M., Frommer, M., Miller, O. J. and Macleod, D. (1985) *Cell*, **40**(1), 91-9.

2. Cedar, H. (1988) *Cell*, **53**(1), 3-4.

3. Constancia, M., Pickard, B., Kelsey, G. and Reik, W. (1998) *Genome Res*, **8**(9), 881-900.

4. Heard, E., Clerc, P. and Avner, P. (1997) *Annu Rev Genet*, **31**, 571-610.

5. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E. and Baylin, S. B. (1994) *Nat Genet*, **7**(4), 536-40.

6. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. and Wolffe, A. P. (1998) *Nat Genet*, **19**(2), 187-91.

7. Weinberg, R. A. (1995) *Ann N Y Acad Sci*, **758**, 331-8.

8. Laird, P. W. and Jaenisch, R. (1994) *Hum Mol Genet*, **3**(Spec No), 1487-95.

9. Klein, C. B. and Costa, M. (1997) *Mutat Res*, **386**(2), 163-80.

10. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E. and Baylin, S. B. (1995) *Cancer Res*, **55**(22), 5195-9.

11. Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B. and Baylin, S. B. (1992) *Proc Natl Acad Sci U S A*, **89**(5), 1929-33.

12. Makos, M., Nelkin, B. D., Reiter, R. E., Gnarra, J. R., Brooks, J., Isaacs, W., Linehan, M. and Baylin, S. B. (1993) *Cancer Res*, **53**(12), 2719-22.

13. Makos, M., Nelkin, B. D., Chazin, V. R., Cavenee, W. K., Brodeur, G. M. and Baylin, S. B. (1993) *Cancer Res*, **53**(12), 2715-8.

14. Nelson, J. B., Lee, W. H., Nguyen, S. H., Jarrard, D. F., Brooks, J. D., Magnuson, S. R., Opgenorth, T. J., Nelson, W. G. and Bova, G. S. (1997) *Cancer Res*, **57**(1), 35-7.

15. Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M. and Kolodner, R. (1997) *Cancer Res*, **57**(5), 808-11.

16. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and et al. (1995) *Nucleic Acids Res*, **23**(21), 4407-14.

17. McClelland, M. and Ivarie, R. (1982) *Nucleic Acids Res*, **10**(23), 7865-77.

18. Yoshikawa, H., de la Monte, S., Nagai, H., Wands, J. R., Matsubara, K. and Fujiyama, A. (1996) *Genomics*, **31**(1), 28-35.

19. Clark, S. J., Harrison, J., Paul, C. L. and Frommer, M. (1994) *Nucleic Acids Res*, **22**(15), 2990-7.

20. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D. and Baylin, S. B. (1996) *Proc Natl Acad Sci U S A*, **93**(18), 9821-6.

21. Gonzalgo, M. L. and Jones, P. A. (1997) *Nucleic Acids Res*, **25**(12), 2529-31.

22. Xiong, Z. and Laird, P. W. (1997) *Nucleic Acids Res*, **25**(12), 2532-4.

23. Jarrard, D. F., Bova, G. S., Ewing, C. M., Pin, S. S., Nguyen, S. H., Baylin, S. B., Cairns, P., Sidransky, D., Herman, J. G. and Isaacs, W. B. (1997) *Genes Chromosomes Cancer*, **19**(2), 90-6.

24. Herman, J. G., Jen, J., Merlo, A. and Baylin, S. B. (1996) *Cancer Res*, **56**(4), 722-7.

25. Waber, P. G., Lee, N. K. and Nisen, P. D. (1996) *Oncogene*, **12**(2), 365-9.

26. Singhal, R. P., Mays-Hoopes, L. L. and Eichhorn, G. L. (1987) *Mech Ageing Dev*, **41**(3), 199-210.

27. Huang, T. H., Laux, D. E., Hamlin, B. C., Tran, P., Tran, H. and Lubahn, D. B. (1997) *Cancer Res*, **57**(6), 1030-4.

28. Hatada, I., Sugama, T. and Mukai, T. (1993) *Nucleic Acids Res*, **21**(24), 5577-82.

29. Reyna-Lopez, G. E., Simpson, J. and Ruiz-Herrera, J. (1997) *Mol Gen Genet*, **253**(6), 703-10.

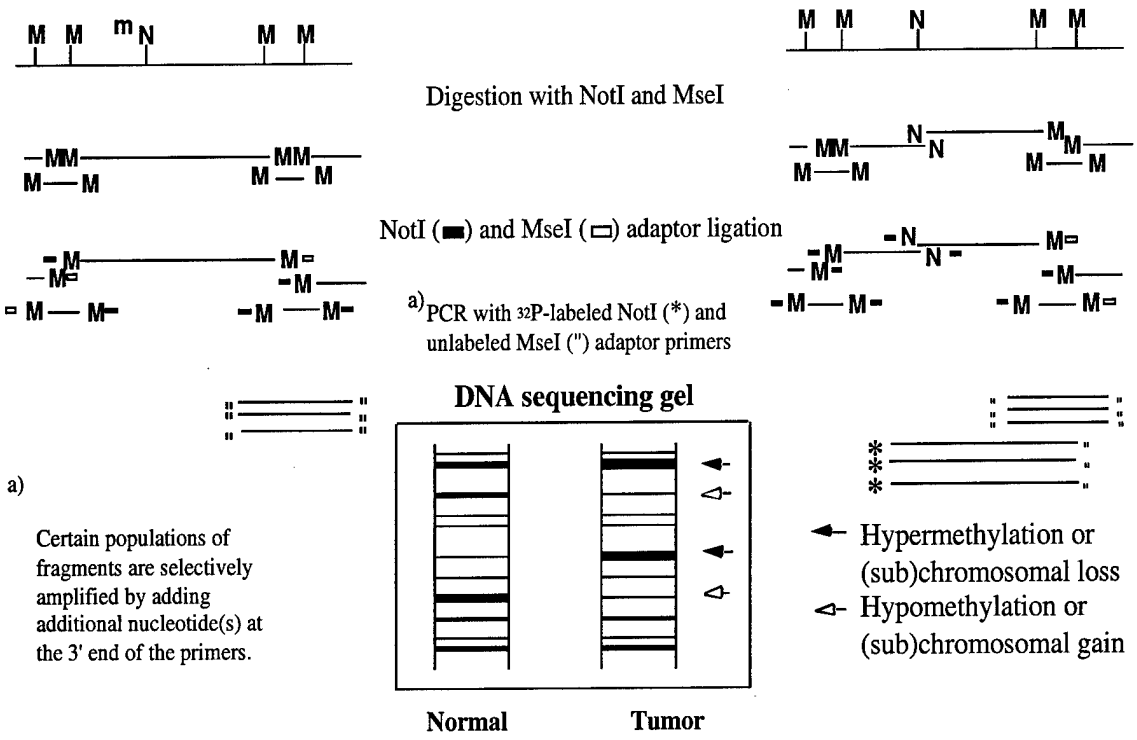
30. Akama, T. O., Okazaki, Y., Ito, M., Okuizumi, H., Konno, H., Muramatsu, M., Plass, C., Held, W. A. and Hayashizaki, Y. (1997) *Cancer Res*, **57**(15), 3294-9.

31. Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M. and et al. (1994) *Nat Genet*, **6**(1), 33-40.

32. Xiong, L. Z., Xu, C. G., Saghai Maroof, M. A. and Zhang, Q. (1999) *Mol Gen Genet*, **261**(3), 439-46.

Figure 1

NotI-MseI MS-AFLP



a) Certain populations of fragments are selectively amplified by adding additional nucleotide(s) at the 3' end of the primers.

Figure 2

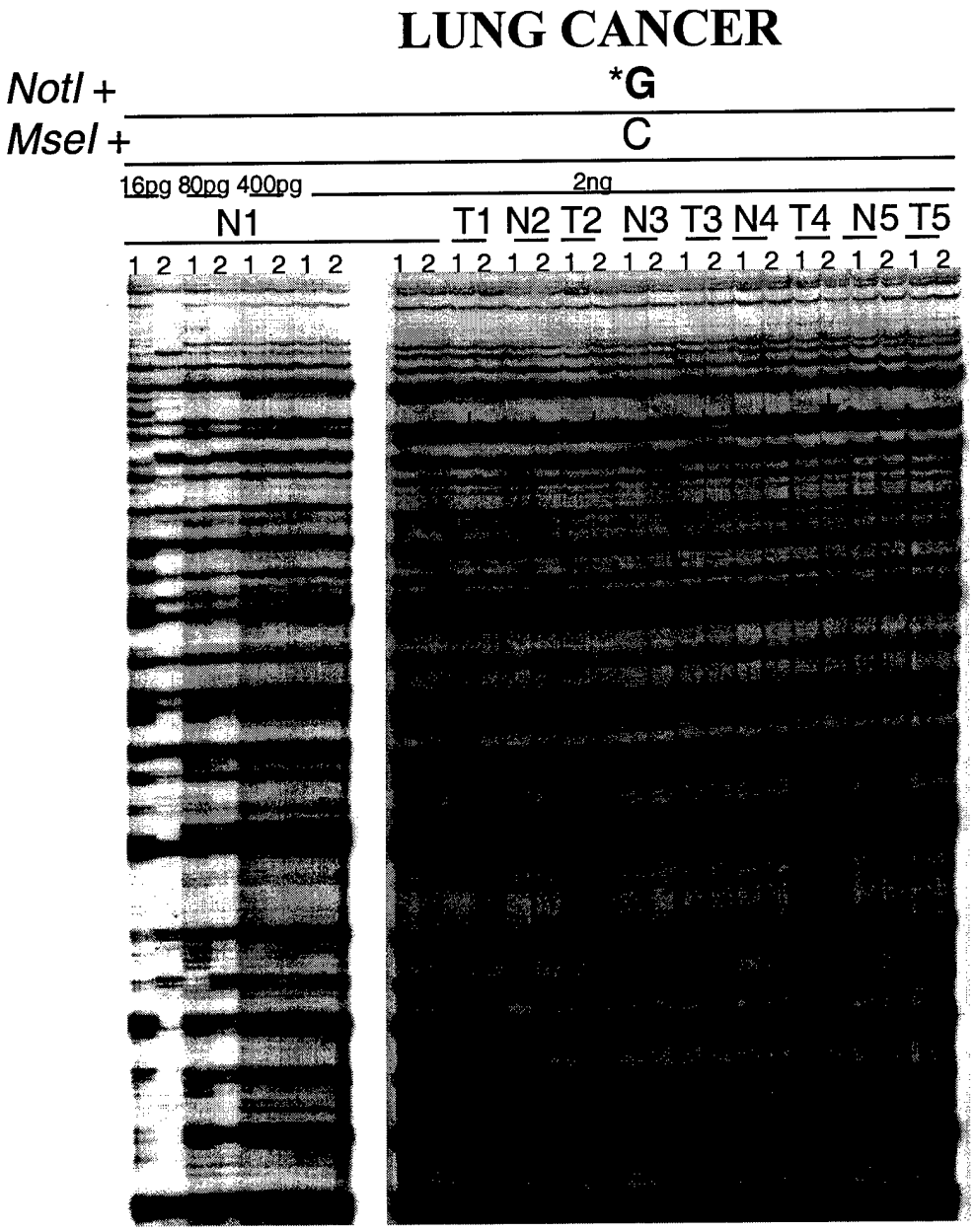

LUNG CANCER
NotI +
*G
MseI +
C
16pg 80pg 400pg
2ng
N1
T1 N2 T2 N3 T3 N4 T4 N5 T5
1 2 1 2 1 2 1 2
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2

Figure 3

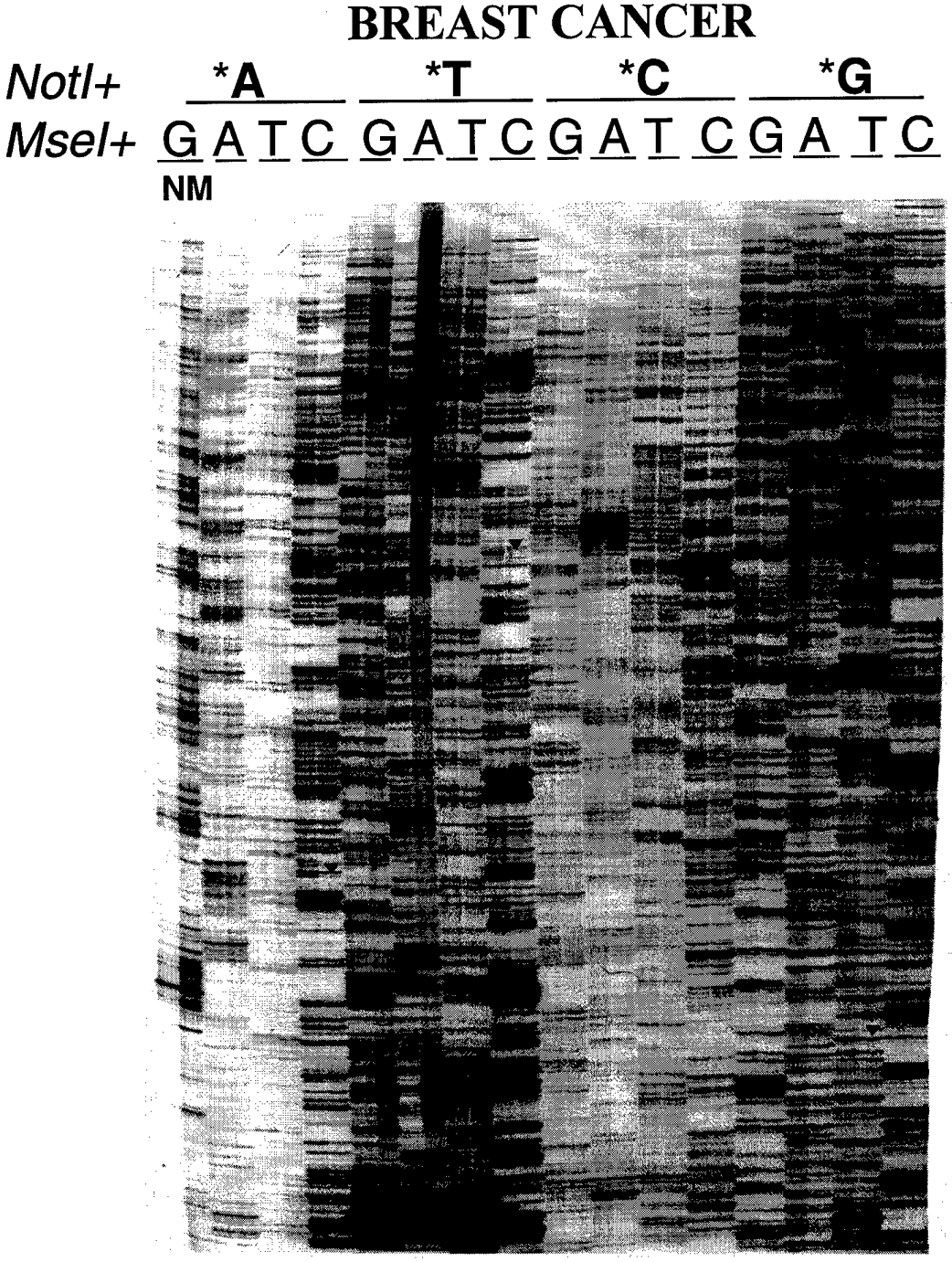


Figure 4

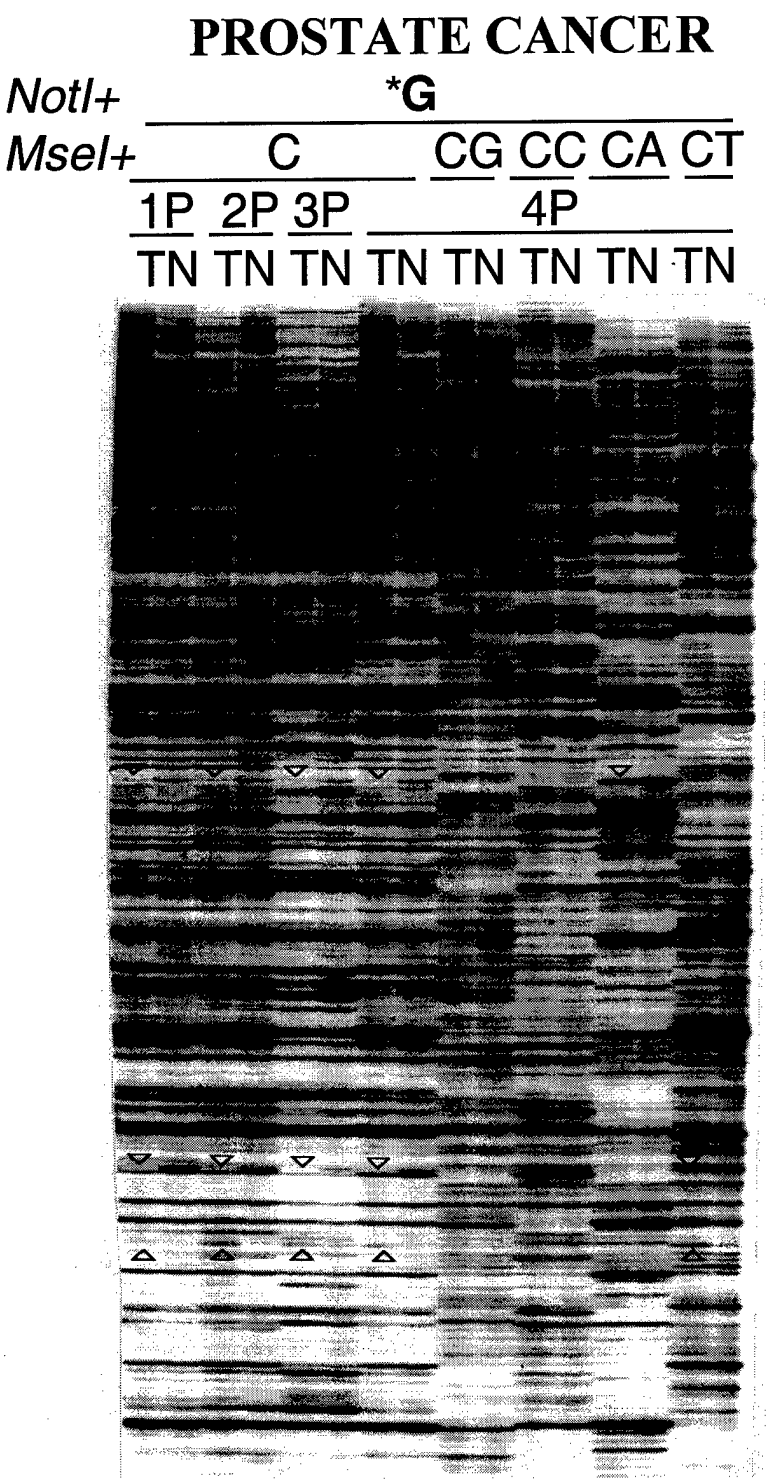

PROSTATE CANCER
NotI+ *G
MseI+ C CG CC CA CT
1P 2P 3P 4P
TN TN TN TN TN TN TN TN

Figure 5

BREAST CANCER

NotI+ G *G G

MseI+ *G G A C T *T

N N M N M N M N M M

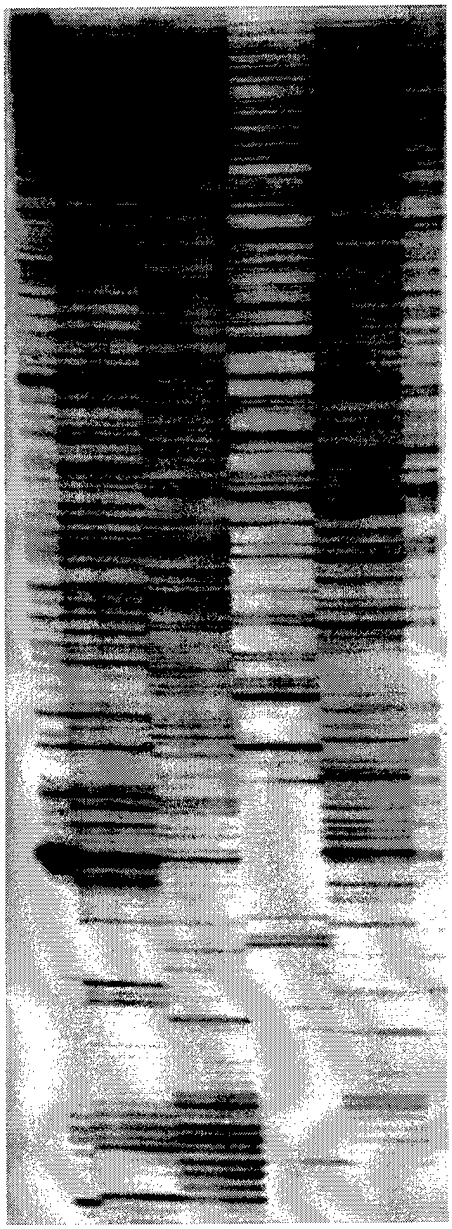


Figure 6